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(54) Title: EXPRESSION OF GENES IN TRANSGENIC PLANTS

(57) Abstract

DNA construct for use in transforming plant cells which comprises an exogenous coding sequence with upstream promoter and downstream terminator sequences, the promoter being a DNA sequence homologous to the DNA control sequence found upstream of a gene involved in carotenoid biosynthesis, for example the gene encoding phytoene synthase. The invention also includes plant cells containing such constructs and plants derived therefrom. Plants according to the invention may be stimulated to express the exogenous coding sequences by application of ethylene.

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EXPRESSION OF GENES IN TRANSGENIC PLANTS

The present invention relates to the expression of genes in transgenic plants. In particular it is concerned with the isolation and use of DNA sequences which control the expression of foreign genes in ripening fruits and in response to ethylene.

The ability to isolate and manipulate plant genes has opened the way to gain understanding about the mechanisms involved in the regulation of plant gene expression. This knowledge is important for the exploitation of genetic engineering techniques to applied problems such as the expression of genes in genetically manipulated crop plants exhibiting improved quality and production characteristics. Many examples are now in the literature of plant DNA sequences which have been used to drive the expression of foreign genes in plants. In most instances the regions immediately 5' to the coding regions of genes have been used in gene constructs. These regions are referred to as promoter sequences. They may be derived from plant DNA; or from other sources, eg, viruses. It has been demonstrated that sequences up to 500-1000 bases in most instances are sufficient to allow for the regulated expression of foreign genes. This regulation has involved tissue-specificity, regulation by external factors such as light, heat treatment, chemicals, hormones, and developmental regulation. However, it has also been shown that sequences much longer than 1 kb may have useful features which permit high levels of gene expression in transgenic plants.

These experiments have been carried out using gene fusions between the promoter sequences and foreign genes such as bacterial promoter genes, etc. This has led to the identification of useful promoter sequences. In work leading to the present invention we have identified a gene which expresses an enzyme involved in the ripening of

tomatoes. We have now shown that it is involved in carotenoid synthesis. The gene in question is encoded (almost completely) in the clone pTOM5, disclosed by Ray et al (Nucleic Acids Research, 15, 10587, 1989). Hereinafter this gene is referred to as the phytoene synthase (or PS) gene; the enzyme for which the pTOM5 gene codes is the pTOM5 gene product. We have shown that the pTOM5 gene is involved in the step or steps of the pathway between geranylgeranyl pyrophosphate and phytoene, and that the pTOM5 gene product is the enzyme known as phytoene synthase. Among the products produced by this branch of the pathway are carotenes, lutein, xanthophylls, and pigments such as lycopene, as well as plant growth regulators such as IBA. We have now isolated a part of the chromosomes of tomato in which the pTOM5 gene is localised. We now disclose the structure of this gene and its transcriptional control sequences, in particular its promoter.

Evidence for the involvement of the pTOM5 gene product in carotenoid synthesis has come from experiments in which the expression of the pTOM5 gene has been inhibited using antisense RNA (see PCT patent application 90/01924). The resulting plants have fruit which are yellow and lack lycopene, indicating that lycopene synthesis has been inhibited. Biochemical precursor feeding experiments have shown that geranylgeranyl pyrophosphate accumulates in extracts of these fruit, indicating that phytoene synthase is inhibited.

Further evidence for the function of the pTOM5 gene in the carotenoid pathway is the significant degree of homology (27% identity; 17% similarity) between the polypeptide predicted from the translation of the open sequence in the clone pTOM5 and the protein encoded by the crtB gene from Rhodobacter capsulatus, a gram-negative purple non-sulphur bacterium. The crtB gene product catalyses the tail-to-tail dimerisation of geranylgeranyl

diphosphate to form prephytoene diphosphate. This enzyme (phytoene synthase) is the point of divergence of carotenoid biosynthesis from other isoprenoid metabolism. Further, an enzyme has been isolated from Capsicum annuum fruit chromoplasts which is believed to catalyse both the synthesis of prephytoene diphosphate and its subsequent conversion to phytoene. This enzyme has a molecular weight of 47,500, in close agreement with the predicted size of the pTOM5 gene product (48,000). The final conclusion comes from complementation experiments in which pTOM5 cDNA has been used to complement an Erwinia mutant which is deficient in phytoene synthase.

We have shown that phytoene synthase mRNA is expressed in ripening tomato fruit. No expression could be detected in green fruit. The phytoene synthase gene is expressed most strongly at the full orange stage of ripening. The level of mRNA then declines in line with the general decline in biosynthetic capacity of the ripening fruit. Expression of phytoene synthase mRNA could also be induced by exposing mature green fruit to exogenous ethylene. The expression of the phytoene synthase gene is reduced in the Ripening Inhibitor (rin) and Neverripe (Nr) tomato fruit ripening mutants, which mature very slowly and never achieve the full red colour of ordinary tomato fruit.

The genomic locations in the tomato of sequences homologous to the pTOM5 clone have been identified using RFLP mapping: two loci, on chromosome 2 and chromosome 3 respectively, carry sequences homologous to the pTOM5 clone. It has also been shown by Southern blotting that the pTOM5 gene may be present as a small multigene family.

The present invention proposes to use the promoters of the phytoene synthase and similar genes to control the expression of novel and exogenous proteins and genes in tomato fruit.

According to the present invention we provide a DNA construct for use in transforming plant cells which

comprises an exogenous coding sequence under the control of upstream promoter and downstream terminator sequences, characterised in that the upstream promoter has homology to a promoter of a gene of the carotenoid biosynthesis pathway. We further provide novel plant cells, and plants, particularly tomatoes, transformed with constructs according to the present invention.

We further provide a process for stimulating the expression of exogenous coding sequences in plants by applying ethylene to plants transformed with constructs according to the invention.

Promoters for use in the invention may be derived from genes such as phytoene desaturase, cyclase and epoxydase. Such promoters may be isolated from genomic libraries by the use of cDNA probes, as has been done in the case of pTOM5. We particularly prefer to use the promoter of the phytoene synthase gene.

The downstream (3') terminator sequences can also be derived from the phytoene synthase gene: or they can be derived from other genes such as the polygalacturonase gene (see UK Patent Application 9025323.9 filed 8 November 1990). Many other possibilities are available from the literature.

By the term 'exogenous coding sequence' we indicate a sequence of DNA, other than that which follows the promoter region in the natural pTOM5 gene, that is adapted to be transcribed into functional RNA under the action of plant cell enzymes such as RNA polymerase. Functional RNA is RNA which affects the biochemistry of the cell: it may for example be mRNA which is translated into protein by ribosomes; or antisense RNA which inhibits the translation of mRNA complementary (or otherwise related) to it into protein. In principle all kinds of exogenous coding sequences are useful in the present invention.

Where the exogenous coding sequence codes for mRNA for a protein, this protein may be of bacterial origin

(such as enzymes involved in polysaccharide metabolism and cell wall metabolism), of eukaryotic origin (such as pharmaceutically active polypeptides) or of plant origin (such as the product of the phytoene synthase gene itself, enzymes involved in respiration, ethylene synthesis, sugar metabolism, aroma and flavour production and cell wall metabolism), or genes or parts thereof in sense and antisense orientation. Of particular interest is the ability of the phytoene synthase gene promoter to respond to exogenously supplied ethylene.

A wide variety of exogenous coding sequences is known from the literature, and the present invention is applicable to these as well as many others yet to be reported. As well as functional mRNA, the exogenous gene may code for RNA that interferes with the function of any kind of mRNA produced by the plant cell: for example, antisense RNA complementary to mRNA for fruit ripening genes such as polygalacturonase, pectinesterase, β-1,4-glucanase, pTOM13 etc.

The construction of these vectors and constructs is described in more detail in the Examples below. For convenience it will be generally found suitable to use promoter sequences (upstream - i.e. 5′ - of the coding sequence of the gene) of between 100 and 2000 bases in length.

Plant cells according to the invention may be transformed with constructs of the invention according to a variety of known methods (Agrobacterium Ti plasmids, electroporation, microinjection, microprojectile gun, etc). The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way, although the latter are usually more easy to regenerate.

Examples of genetically modified plants according to

the present invention include, as well as tomatoes, fruits such as mangoes, peaches, apples, pears, strawberries, bananas and melons; and field crops such as maize (corn), sunflowers, sugarbeet, canola, and smallgrain cereals such as wheat, barley and rice.

Plants produced by the process of the invention may contain more than one recombinant construct. As well as one or more constructs containing the phytoene synthase promoter, they may contain a wide variety of other recombinant constructs, for example constructs having different effects on fruit ripening. In particular where the invention is applied to tomatoes, these may be of enhanced colour (as a result of inserting extra gene copies of the PS gene and thereby overexpressing phytoene synthase) and may also contain constructs inhibiting the production of enzymes such as polygalacturonase and pectinesterase, or interfering with ethylene production (eg from pTOM13, see PCT Application 90/01072 filed 12 July 1990). Such tomatoes can have higher solids contents than conventional tomatoes and produce more tomato paste per unit of fruit weight. The extra lycopene production in such tomatoes is desirable to prevent any lightening of colour that might otherwise be observed in such pastes. Tomatoes containing more than one type of recombinant construct may be made either by successive transformations, or by successively crossing varieties that each contain one of the constructs, and selecting among the progeny for those that contain all the desired constructs.

A further aspect of the present invention is a process of activating exogenous coding sequences in plants under the control of the phytoene synthase promoter which comprises the application of exogenous ethylene. This may find particular use when fruit is stored in the absence of ethylene, and ethylene is then used to switch on the production of a given useful character providing extra value to the fruit at the point of sale. This may lead to

increase in sweetness of the fruit, or the production of special flavours or aromas, or the production of special polypeptides desired by the consumer. This will enable more flexibility in control of the fruit ripening process, particularly at the point of sale.

We now describe the isolation of genomic clones from a tomato library encoding the phytoene synthase gene and related sequences. Genomic clones representing two individual genes have been isolated and characterised by DNA sequence analysis. The clone gTOM5 represents part of a gene with exon sequence identical to the clone pTOM5. Clone F contains a sequence similar but not identical to pTOM5. Details of these clones are given below. Sequence and expression data suggest that Clone F encodes an untranscribed pseudogene. The genomic clones described in the Examples cover most of the coding region and the complete transcriptional initiation region of the phytoene synthase gene. The clone gTOM5 has been deposited at the National Collections of Industrial and Marine Bacteria (NCIB), now at 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland, on 11 March 1991 under the reference NCIB Number 40382 while pTOM5 has been deposited at NCIB as a plasmid in E.coli, under the reference NCIB 40191, on 1 September 1989.

The invention will be further described with reference to the following drawings, in which:

Figures 1 and 1A show the nucleotide sequence of the 3.5 kb EcoRI - SalI fragment of gTOM5 (SEQ ID: 1) and the 3' region of the phytoene synthase gene (SEQ ID: 2);

Figure 2 is a diagram of the structure of the phytoene synthase gene;

Figure 3 outlines a scheme for polymerase chain reaction amplification of the phytoene synthase gene promoter fragment;

Figure 4 outlines a scheme for construction of the plant transformation vector p5TAK.

EXAMPLE 1

1.1 Isolation of pTOM5 related genes

A library was constructed from tomato (Lycopersicon esculentum var. Ailsa Craig) genomic DNA which was partially digested with Sau3A and cloned into lambda EMBL3 (Bird et al (1988) Plant Molecular Biology 11, 651-662). The library was screened with the pTOM5 cDNA insert (Ray et al (1987) Nucleic Acids Research 15, 10587) and positive phages were purified by four successive cycles of plaque purification. Five positive clones were isolated.

Restriction fragment mapping and DNA sequence analysis of these clones indicated that all 5 clones were overlapping and related. The clones did not have 100% sequence homology to pTOM5 in the regions that probably represented exons. This indicated that these clones represented a gene (designated clone F) that was not the pTOM5 gene.

In order to isolate the phytoene synthase gene, synthetic oligonucleotides were designed that hybridised specifically to either pTOM5 or the clone F. The sequences of oligonucleotides CL100 and CL99 represented a region where the pTOM5 sequence is only 54% homologous to the sequence of clone F:

CL100 - 5'-CATCTGTTCCGATGTCATCGTCCG-3' pTOM5 specific
CL99 - 5'-TTTTTTTTCTGATGACACAGCCAT-3' clone F specific

CL100 was used to screen the same genomic library. After four rounds of purification, one phage (designated GTOM5) was isolated which hybridised to CL100 and pTOM5 but not to CL99.

1.2 Characterisation of the phytoene synthase gene promoter sequence

A 3.5 kb EcoRI - SalI fragment was isolated from GTOM5 and the complete nucleotide sequence of fragment has been determined (Fig 1). This sequence contained exon regions that were 100% homologous to pTOM5 but did not contain the 3' end of the cDNA (Fig 2). The fragment contained 1.1 kb of sequence extending 5' of the end of the cDNA. This sequence represents the pTOM5 gene promoter.

1.3 Isolation and characterisation of the 3' region of the phytoene synthase gene

Synthetic oligonucleotides were designed for use as primers for polymerase chain reaction (PCR) amplification of a specific fragment containing the 3' region of the pTOM5 gene with BamHI restriction sites at each end. The oligonucleotides (designated 5GENE-5 and 5GENE-3) contain sequences from base 3405 to 3442 of SEQ ID:1 and 1604 to 1630 of the pTOM5 cDNA.

After PCR followed by BamHI digestion, two fragments (approximately 800 and 570 bp) were identified by agarose gel electrophoresis. These fragments were isolated, restricted with BamHI and cloned into M13mp18. Clones containing each fragment were identified and the nucleotide sequence was determined (Fig 1).

1.4 Isolation of a phytoene synthase gene promoter fragment

Synthetic oligonucleotides were designed for use as primers for polymerase chain reaction (PCR) amplification of a specific fragment containing the phytoene synthase gene promoter with restriction sites at each end (5'- HindIII : 3'- BamHI). The oligonucleotides (designated 5PRO-5 and 5PRO-3) contain sequences from base 1 to 30 and 1155 to 1105 of the phytoene synthase gene:

```
                          1                                  30
5PRO-5 TCGAAGTCAGAAGCTTGAATTCATAAACTTTAAATTTTAAATTTTG
                  HindIII
    1155                                                  1105
5PRO-3 CAAACAAAGGATCCCACTTTCTCTTCTGTAGAAAAAGATTATAAAAAGACC
                  BamHI
```

These primers were used in a PCR with tomato genomic DNA (Lycopersicon esculentum var. Ailsa Craig) to amplify a 1171 bp fragment that contained the phytoene synthase gene promoter sequence and 52 bp of the 5' untranslated region of pTOM5 (Fig 3). This fragment was digested with HindIII and BamHI and cloned into M13mp18. The nucleotide sequence of one clone (p5PRO) was found to be identical to that of the same region of GTOM5.

1.5 Construction of plant transformation vector - p5TAK

The 1151 bp HindIII/BamHI phytoene synthase gene promoter fragment from the M13mp18 clone (p5PRO) is excised from replicative form DNA and cloned into HindIII and BamHI cut pTAK1 (described in EP 271988 A). Plasmids with the correct orientation of the PS gene promoter are identified by restriction analysis and DNA sequencing. One such clone is designated p5TAK (Fig 4).

EXAMPLE 2

Generation of transformed plants

The vector p5TAK (from Example 1.5) is transferred to Agrobacterium tumefaciens LBA4404 (a micro-organism widely available to plant biotechnologists) and is used to transform tomato plants. Transformation of tomato stem segments follows standard protocols (eg. Bird et al Plant Molecular Biology 11, 651-662, 1988). Transformed plants are identified by their ability to grow on media containing the antibiotic kanamycin. Plants are regenerated and grown to maturity.

The ripening-specific expression of the β-glucuronidase (GUS) gene as determined by the phytoene synthase gene promoter is demonstrated by analysis of mature green, breaker and ripening fruit for GUS enzyme activity. The response of the gene to exogenous ethylene is demonstrated by incubation of breaker stage fruit in an atmosphere containing additional ethylene followed by analysis of GUS enzyme activity.

CLAIMS

1. A DNA construct for use in transforming plant cells which comprises an exogenous coding sequence under the control of upstream promoter and downstream terminator sequences, characterised in that the upstream promoter has homology to a promoter of a gene of the carotenoid biosynthesis pathway.

2. A DNA construct as claimed in claim 1 in which the gene of the carotenoid biosynthesis pathway is the phytoene synthase gene.

3. A DNA construct as claimed in claim 2 in which the exogenous coding sequence codes for RNA that inhibits expression of a plant gene.

4. A DNA construct as claimed in claim 3 in which the exogenous coding sequence is antisense to part of the coding strand of a plant gene.

5. A DNA construct as claimed in claim 1 in which the exogenous coding sequence codes for mRNA that is translated into an enzyme functional in plants.

6. A DNA construct claimed in any of claims 2 to 4 in which the upstream promoter is homologous to the sequence shown in Figure 1.

7. A DNA construct claimed in claim 6 in which the upstream promoter is a DNA sequence homologous to at least 100 bases of the sequence shown in Figure 1.

8. Plant cells transformed with DNA constructs claimed in any of claims 1 to 7.

9. Plants comprising cells as claimed in claim 8.

10. Plants as claimed in claim 8 which are tomatoes, mangoes, peaches, apples, pears, strawberries, bananas or melons.

11. A process for stimulating the expression of exogenous coding sequences in plant cells by applying ethylene to plant cells claimed in claim 8.

12. A process as claimed in claim 11 in which the plant cells form part of a growing plant.

13. A process as claimed in claim 11 in which the plant cells form part of harvested material.

14. A process as claimed in any of claims 11-13 in which the exogenous coding sequences express mRNA that is translated into protein functional in the plant cell.

15. A process as claimed in claim 14 in which the protein is a fruit ripening enzyme.

SEQ ID NO: 1
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 3485bp

FIG.1 (1/3)

STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: DNA

ORIGINAL SOURCE ORGANISM: TOMATO var. Ailsa craig
IMMEDIATE EXPERIMENTAL SOURCE: EMBL clone GTOM5

FEATURES:
from 1 to 1091bp promoter region
from 1092 to 1703bp exon A'
from 1831 to 1881bp exon D'
from 2301 to 2480bp exon E'
from 2790 to 3025bp exon F'

PROPERTIES: Fragment of gene coding cDNA pTOM5 exons A' to F'

FIG. 1 (2/3)

```
GAATTCATAA ACTTTAAATT TTAAATTTTG ACTTCGCTTT GTGTGACATA TCAATTACAG     60
AAATTCAGAG TGGCCATTGT TGAAAGAGAG GGTGGAATTT GTGTAAGTTT TGTTTCCTTT    120
CAGTTCTTGA TATATAAAGT TGCAATCTTT AACATTCTTT GTTCACTTTC TATAGGTTTG    180
CTAGGTTCGG TTAAATTCAG TAGCTTTAGT TTAAACCCTA TGCGGAATAG AGAATGTGTA    240
AACTTTAAAC TTCAAATTTT GGCTCCGCAT ACGACTAGCG ACTATATAAT AATAGGAATT    300
GAGCACTTGG CTTTTGTATA TAGCTTCTAT GTGTACCAAA ATTAGAAAAT CAGGCGATTA    360
TTATATCTTG TTGACTAAAT ATAGAATGCA TCCATTACCC CCAAAAAGTG TGATTCCACT    420
GTCATAGGAG GTTTTTTTTA TTTCATTTTA TTTGTGCTTT CAATAATGTA GAGTAGTTTA    480
CAAAGATCCT TTCTTTGTGA CACATGGTAG GTAATATTGC TGATTTTAGT TGTAGTTTTG    540
GGGCTTAATA AATGTTCGAA ATTATTTATA CTGAGGTTAC GGGGTTACGG GGTTGTCTAT    600
AAATGCAGGT TATGGTTTTA CGTGAACTCA ATAATTATTG TAGATACTAA GAAATCCACT    660
CAGTGTTCTT GCGGTGTCTT GCTTTTGATT TCAGCATCAC TTGTGAGTTG ATTGTGTTTA    720
GATTATCACA TTATTCTGTG GCTGTAACTG TATCCTTGTT AGTTGCTTTG TTTCTACACT    780
GTTGTTTTCC CTCTTTTATA CCTATTTTGA TGTGTTGTAC TCGAACGAGG GTCATCGGGG    840
AACAACCTCT TTACCTCCGT GAGGTAGAGC TATGGTCTGT GTCCACTCTA CCCTCCCCAG    900
ATCCCTCTTG TAGGATTTCA CTATATTGTA ATATTAACTT GAGGTCACTA TAGGAGCTCA    960
AAAACTTCTA ATTTTGAATC AATGTCTGGT TATACTTTTT TTGTCATAAC TGTATCTCAA   1020
ATGTGGTGTT TGGTTTATCT CATTTTGCAG AAGTCAAGAA ACAGGTTACT CCTATTTGTG   1080
AGGCTAGTCA ATTTGCCTGT CTGTGGTCTT TTTATAATCT TTTTCTACAG AAGAGAAAGT   1140
GGGTAATTTT GTTTGAGAGT GGAAATATTC TCTAGTGGGA ATCTACTAGG AGTAATTTAT   1200
TTTCTATAAA CTAAGTAAAG TTTGGAAGGT GACAAAAAGA AAGACAAAAA TCTTGGAATT   1260
GTTTTAGACA ACCAAGGTTT TCTTGCTCAG AATGTCTGTT GCCTTGTTAT GGGTTGTTTC   1320
TCCTTGTGAC GTCTCAAATG GGACAAGTTT CATGGAATCA GTCCGGGAGG GAAACCGTTT   1380
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FIG.1 (3/3)

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TTTTGATTCA TCGAGGCATA GGAATTTGGT GTCCAATGAG AGAATCAATA GAGGTGGTGG   1440
AAAGCAAACT AATAATGGAC GGAAATTTTC TGTACGGTCT GCTATTTTGG CTACTCCATC   1500
TGGAGAACGG ACGATGACAT CGGAACAGAT GGTCTATGAT GTGGTTTTGA GGCAGGCAGC   1560
CTTGGTGAAG AGGCAACTGA GATCTACCAA TGAGTTAGAA GTGAAGCCGG ATATACCTAT   1620
TCCGGGGAAT TTGGGCTTGT TGAGTGAAGC ATATGATAGG TGTGGTGAAG TATGTGCAGA   1680
GTATGCAAAG ACGTTTAACT TAGGTTAGCT TCTTCAATCT ATTCATTCGT TTACCAAATA   1740
TTATTTGGTA AGCAATAATT ATGAATATAT ATATGTTCAT GTTATTGATG AAGACAAAAT   1800
GTTTTATCGG TGATATTTGA CTTTGATAAG GAACTATGCT AATGACTCCC GAGAGAAGAA   1860
GGGCTATCTG GGCAATATAT GGTGAGGTTC CGCCAGTTTA ATAACAGTTA CGCGCACAAA   1920
CACATATGAT TAATCGGGGG ACGAGAAAAA TAGAAATGAG CTTTGAGTTT TGAGGGGTCA   1980
TATGTAATAG GTAAATCCGA GCTTGACTAG CTTGAGATGT TTATTGTCAT ATCATGCTCA   2040
ATACTAACCA AAACACCTGA AAAAGAACTT GATTACTATT TACATACTAA TTATTTTCAG   2100
TTCTTTGCTG TTCACATTTT TACCTATGGA ACTGGTTTTC GCGATTGTTA TACTTCATAT   2160
TCGATGTTAA TAAAATATAT CATTCCTCCC TTTTTCTCCA CTTCAAGCTT TTACTGTAGT   2220
GTTGAAAGGG GAAACTCCTT TTAATGATTG CATATATAAA CGAATCTTGA GGTTGAATAG   2280
TTTCTCATTA TGATCTGTTT AAACAGTTAT GGTGCAGAAG AACAGATGAA CTTGTTGATG   2340
GCCCAAACGC ATCATATATT ACCCCGGCAG CCTTAGATAG GTGGGAAAAT AGGCTAGAAG   2400
ATGTTTTCAA TGGGCGGCCA TTTGACATGC TCGATGGTGC TTTGTCCGAT ACAGTTTCTA   2460
ACTTTCCAGT TGATATTCAG GTTAGTCTAC CAATTCTATG GTCTTTATAT TGTTCAATTT   2520
CGGTTTGATG TCACTTTGCT GAGGCTTTCT AATAGCTTAC TTCAGCCTAG CGGAAATGTT   2580
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FIG. 1A (1/3)

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TGTAGTTGAA TCTCTAGTTC TGTCTCCTAT ATCTGTTTCT CTCGTCCTAG ATACTACACA 2640
TACTTCATTT CTGTTTTAAC ATTTTATTCG TCTTTTGGTG TTGTTTTGTA TGTGAATCAT 2701
ATATTTGGAA CAGAATCATT ATTAGTTCAC ATGATTTCAT TTGCTTTCTT CAATAGCGTA 2760
ATTGTCTAAC CTTCCAATAT ATGTTGCAGC CATTCAGAGA TATGATTGAA GGAATGCGTA 2820
TGGACTTGAG AAAATCGAGA TACAAAAACT TCGACGAACT ATACCTTTAT TGTTATTATG 2660
TTGCTGGTAC GGTTGGGTTG ATGAGTGTTC CAATTATGGG TATCGCCCCT GAATCAAAGG 2940
CAACAACAGA GAGCGTATAT AATGCTGCTT TGGCTCTGGG GATCGCAAAT CAATTAACTA 3000
ACATACTCAG AGATGTTGGA GAAGAGTAAG TACAAAGCTG TGTTTTACGC ACATAATTTT 3060
TTTTGCTAAT ATTTACATAT CAAAATATAG GAAAATGAGC TCTTCGGTTA TCCGGTTTAT 3120
ATTTTTTTTA TGTCAACATA ATAGTATAAA TGAATTAGTA TGAGTCGTTC TGGGAATAAA 3180
ATTGCAGAAC TCAATTTAGC CGGTGTTGTG AAATCCTGCT GTTTTGAGAG CTTAAAGCTC 3240
ATTAGTTAGT CGTTAGAGAC GAAGAAATTC TTCATTGTGG CCTCTTTATT CCACCTTAAG 3300
TTGTGATATT TTCATTATTG GTACATTTGG CAAAAACACC TGAACAAATT TATGACGGTG 3360
CCTTTTGAAA GTCACTATAC CTGTCTAGTC GGCGTTTTAT CACATTTCTT TGACATATTG 3420
AACTTTGAAA CATGATATCA GCTCTAGACA GTGACGAGCC ATGATCCGTT GACCTGCAGG 3480
TCGAC                                                   3485
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FIG. 1A (2/3)

SEQ ID NO: 2
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 1386bp

STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: DNA

ORIGINAL SOURCE ORGANISM: Tomato var. Ailsa craig
IMMEDIATE EXPERIMENTAL SOURCE: PCR of genomic DNA

FEATURES:
from 1 to 6bp BamHI site introduced by PCR
from 123 to 326bp exon G'
from 1016 to 1380bp exon H'
from 1381 to 1386bp BamHI site introduced by PCR

PROPERTIES: Fragment of gene coding cDNA pTOM5 exons G' to H'

FIG. 1A (3/3)

```
GGATCCGAAC TTTGAAACAT GATATCAGCT CTAGACAGTG ACGAGCCATG ATCAATTTCT    60
TTCCTTTATT CTTTCTTTGG AAGGTGCCGG TATTTAGGCT TCCGTTGTTC TTATATATTG   120
CTTTCCCTGC AGTGCCAGAA GAGGAAGAGT CTACTTGCCT CAAGATGAAT TAGCACAGGC   180
AGGTCTATCC GATGAAGATA TATTTGCTGG AAGGGTGACC GATAAATGGA GAATCTTTAT   240
GAAGAAACAA ATACATAGGG CAAGAAAGTT CTTTGATGAG GCAGAGAAAA GGCGTGACAG   300
AATTGAGCTC AGCTAGTAGA TTCCCTGTAA GCATTCGTAA ACTCTTTAGT TTTATGAAAT   360
GATTCTTTTT TCGCGTTATT AGATGAATAT GGTTGCCTGT GTTGATGTAT TTCTAGGTCG   420
ATGAAGTTGA GACAAGGGTT TTTAAGTTTT AACGACTTTT ACGGGGTGCC ATGTTATCTG   480
CTACCTAATC TTAGGTAGTT GACCGGAAGG GTCTAGAATT TTAACCTCAT GTTCACCCTA   540
CCAACCAAGA AATGAACCTC GCATAGAGCT CGTAGTTATG AATATTTGCT TTGGCATGAC   600
ATTGTGCGGA TCATGAAATG TCTTAGATTA TATGGAAAAA TCATTCTATT ACATCGAATA   660
GATACATTAG ATCTAAGAAG CACGCCGTGT TGTAAATGAG AAATTCTATA GCTCAGATCT   720
TTAGTTTTCT CTGAACGACC TACAAACCAA CGGATAACCT TGTATTGAGC TTGTCGTTCT   780
CAGTATTTGC ACTAACATTA CGTCGTGAGG ATCCTGAAAT GGCTTGGATT GCTATTATTC   840
TGGATATGGC AAAACCATTT TATTAGTACT AGATATCGAA TAACTACATT TGACCCTACA   900
AGTACCCTGG GTTGGAGTAC AATATCCCAT ACCTCGTGTC TTTAGTGTTC TCTTATTTAT   960
CACCTTTGTC TACTATTCTG GCAAAATAAC CTCACTCGTT ACTCGGTGTT TCCAGGTATG  1020
GGCATCTTTG GTCTTGTACC GCAAAATACT AGATGAGATT GAAGCCAATG ACTACAACAA  1080
CTTCACAAAG AGAGCATATG TGAGCAAATC AAAGCAAGTT GATTGCATTA CCTATTGCAT  1140
ATGCAAAATC TCTTGTGCCT CCTACAAAAC TGCCTCTCTT CAAAGATAAA GCATGAAATG  1200
AAGATATATA TATATATATA TATAGCAATG TACATTAGAA GAAAAAAAGG AAGAAGAAAT  1260
GTTGTTGTAT TGATATAAAT GTATATCATA AATATTAGGT TGTAGTAACA TTCAATATAA  1320
TTATCTCTTG TAGTTGTTGT ATCTTCACTT TATCTCAACT CCTTTGAGAG AACTTTCCGT  1380
GGATCC                                                             1386
```

FIG. 2

Structure of the pTOM5 Gene

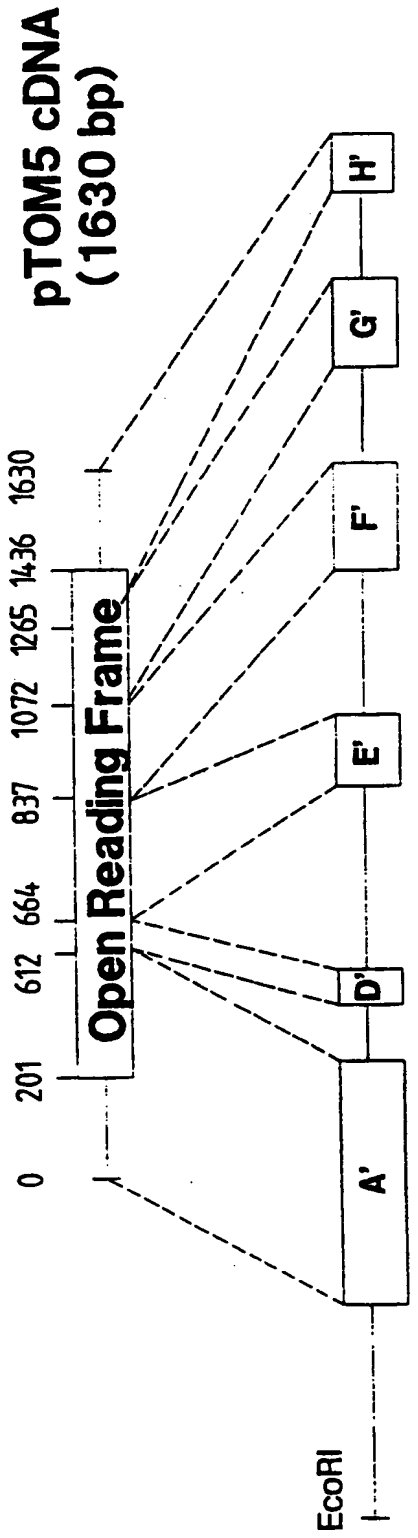


FIG. 3

Polymerase Chain Reaction amplification of a pTOM5 promoter fragment

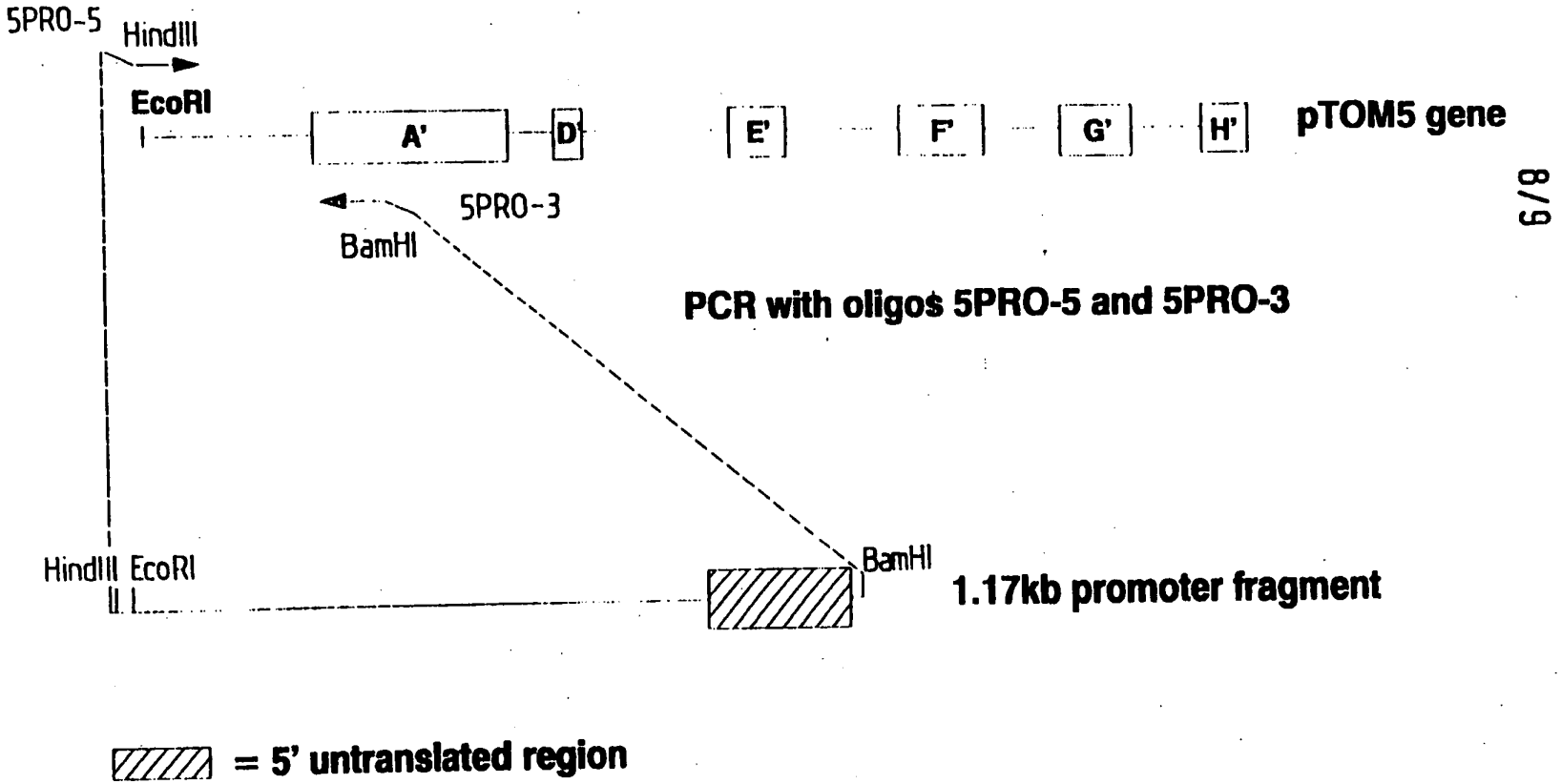
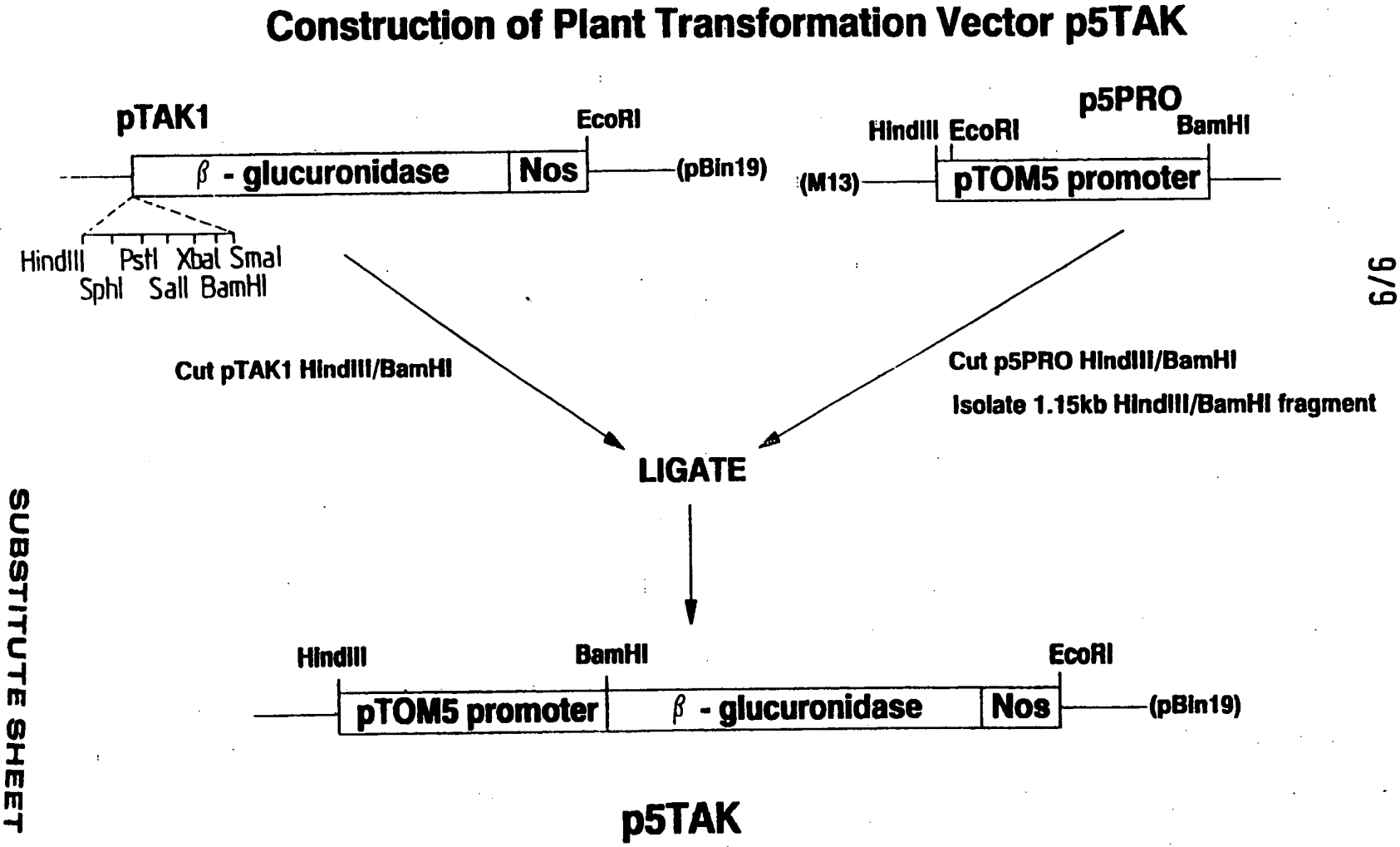


FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00442

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N5/10; C12N15/52

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	NUCLEIC ACIDS RESEARCH. vol. 15, no. 24, 1987, ARLINGTON, VIRGINIA US page 10587; RAY, J., ET AL.: 'Sequence of pTOM5, a ripening related cDNA from tomato' see the whole document ---	1-15
A	PLANT, CELL AND ENVIRONMENT vol. 10, 1987, pages 177 - 184; MAUNDERS, M.J., ET AL.: 'Ethylene stimulates the accumulation of ripening-related mRNAs in tomatoes' see the whole document ---	1-15

-/--

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 JUNE 1992	30. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	THE PLANT CELL. vol. 1, 1989, ROCKVILLE, MD, USA. pages 53 - 69; GIOVANNONI, J. J., ET AL.: 'Expression of a chimeric polygalacturonase gene in transgenic rin (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening' see the whole document	11-15
A	EMBO JOURNAL. vol. 7, no. 11, November 1988, EYNSHAM, OXFORD GB pages 3315 - 3320; DEIKMAN, J., ET AL.: 'Interaction of a DNA binding factor with the 5α-flanking region of an ethylene-responsive fruit ripening gene from tomato' see page 3317	11-15
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A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, December 1990, WASHINGTON US pages 9975 - 9979; ARMSTRONG, G. A., ET AL.: 'Conserved enzymes mediate the early reactions of carotenoid biosynthesis in nonphotosynthetic and photosynthetic prokaryotes' see page 9979, paragraph 2	1-7
A	WO,A,9 101 375 (ICI) 7 February 1991 see page 6, line 6 - line 24	1-7
A	EP,A,0 271 988 (ICI) 22 June 1988 see the whole document	1-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200442 SA 57488

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/06/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9101375	07-02-91	AU-A- 6042390	22-02-91
		EP-A- 0482053	29-04-92
EP-A-0271988	22-06-88	AU-A- 7435091	11-07-91
		AU-A- 8095687	12-05-88
		JP-A- 63164892	08-07-88
		US-A- 5073676	17-12-91